Supplemental Data

Steady-state production of IL-4 modules immunity in mouse strains and is determined by lineage diversity of *i*NKT cells

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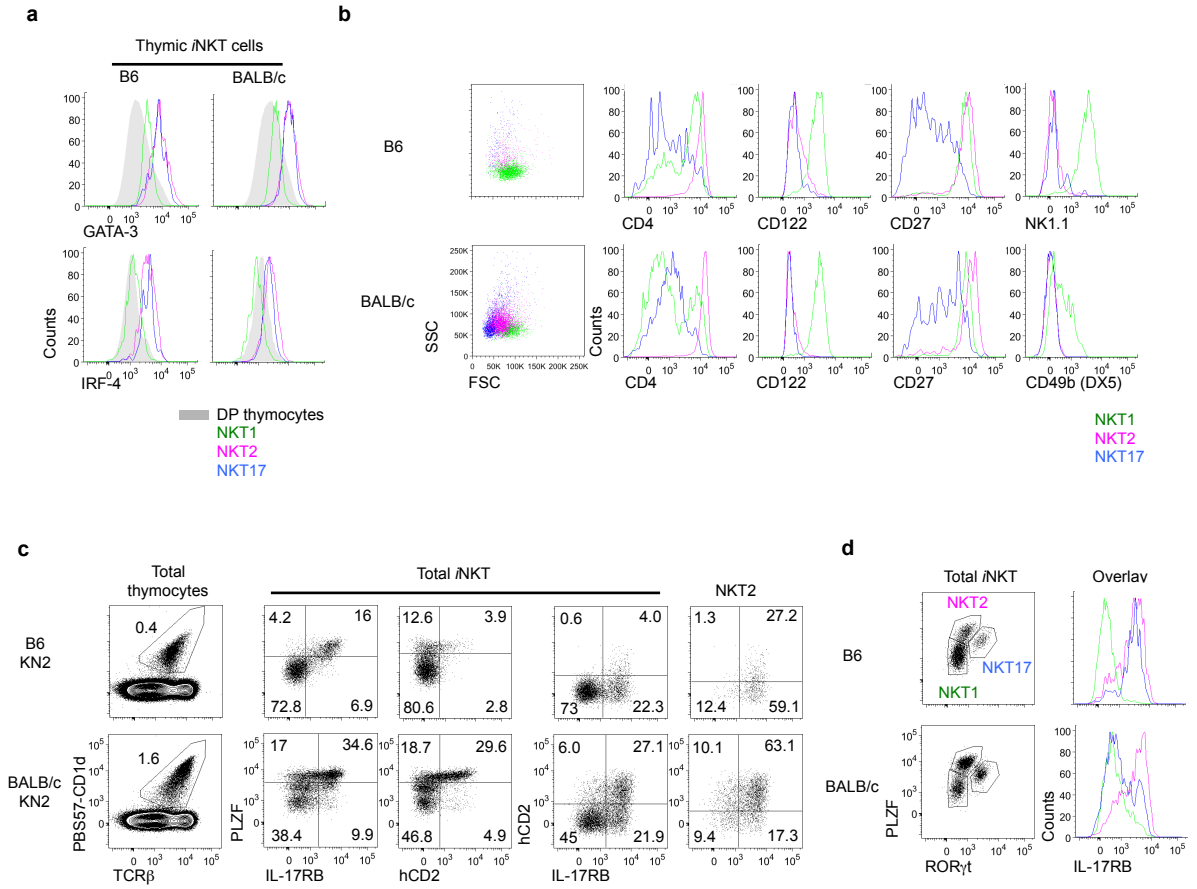
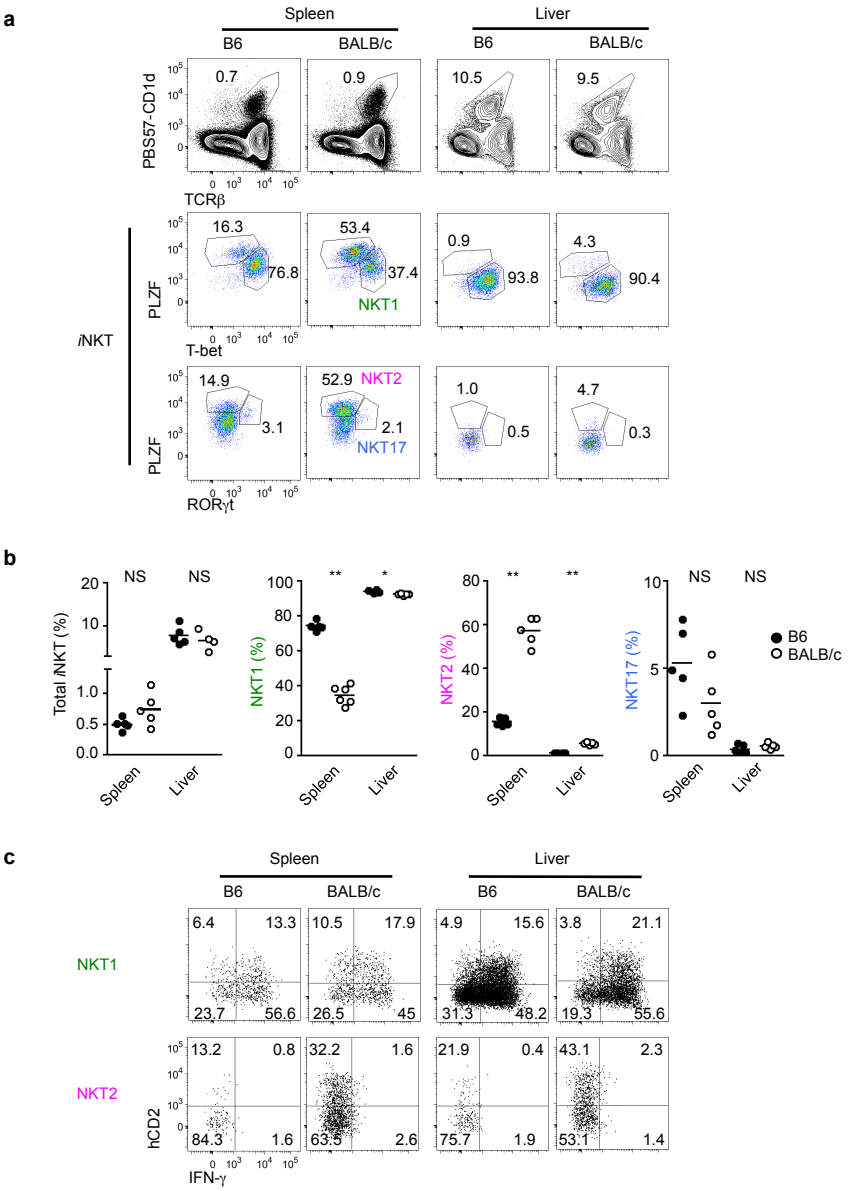
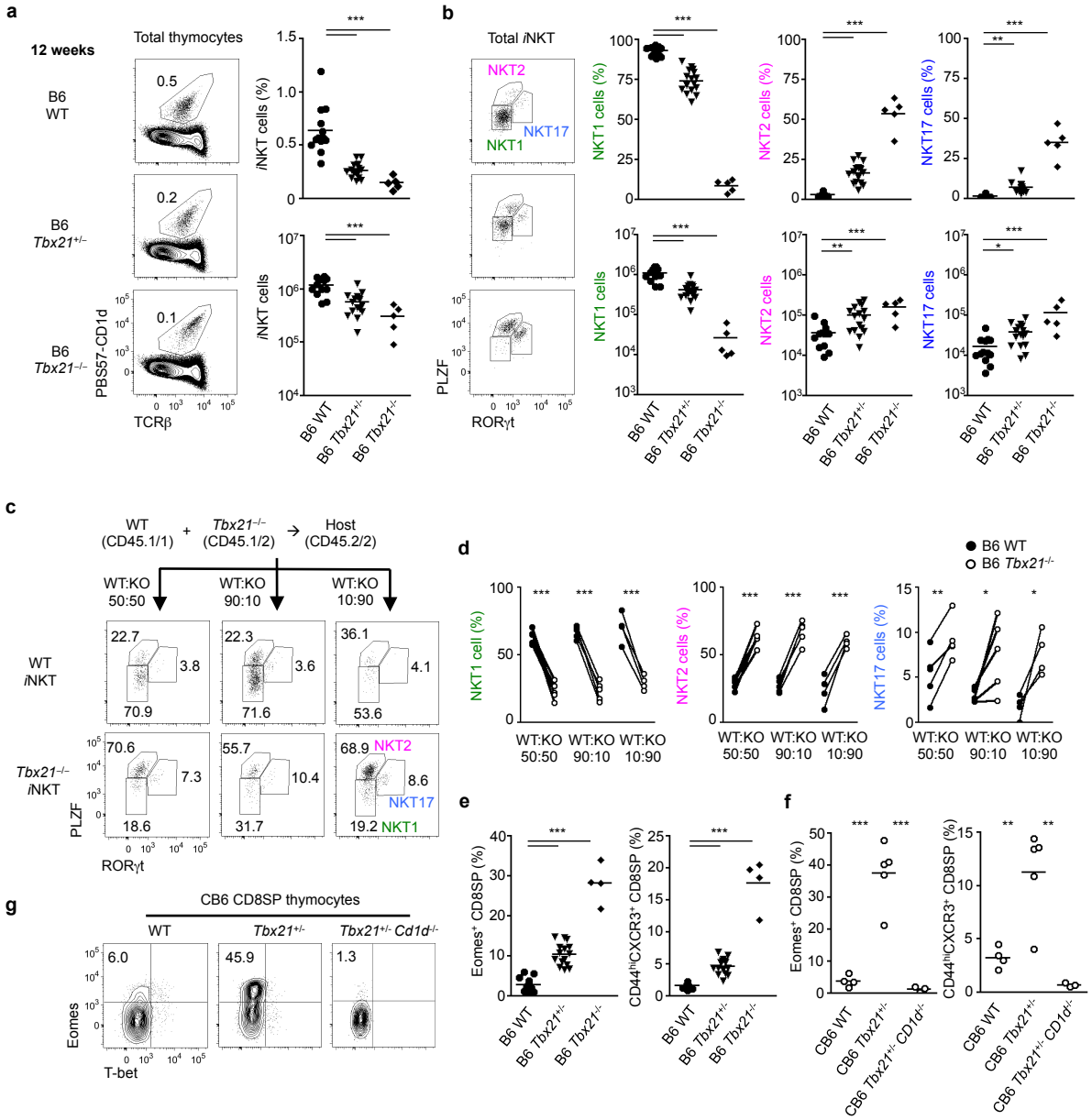


Supplementary Figure 1. GATA-3, IRF-4 and surface markers expression profiles in subsets of *i*NKT cells. **(a)** Thymic *i*NKT cells from 7 week-old B6 and BALB/c mice were intracellularly stained with GATA-3 and IRF-4. Expression on NKT1, NKT2 and NKT17 subsets, as defined in Fig. 2a is shown. Histogram plots of each subset were overlaid with that of DP thymocytes (grey). **(b)** *i*NKT cells from 7 week-old B6 or BALB/c mice were analyzed for the forward and side scatter profile with expression patterns of CD4, CD122, CD27 and NK1.1 or DX5. **(c)** Thymic *i*NKT cells from B6 and BALB/c KN2 mice were stained with PLZF, IL-17RB and hCD2. **(d)** Thymic *i*NKT cells from B6 and BALB/c mice were stained with PLZF, RORγt and IL-17RB. Representative data from three independent experiments are shown (a ~ d).



Supplementary Figure 2. *i*NKT cell phenotype in spleen and liver. **(a)** *i*NKT cells from spleen and liver of 7 week-old WT B6 and BALB/c mice were stained with PLZF, T-bet and RORγt. Representative data of five independent experiments are shown. **(b)** Statistical analysis of percent *i*NKT cells among total cells (left panel) or each iNKT subset among total *i*NKT cells (middle and right panels) in spleen and liver of 6 to 8 week-old mice is shown (n = 4~6). Each symbol represents an individual mouse and unpaired two tailed t-tests were used for comparison. Pooled data from five independent experiments. ** P<0.0001, * P=0.015; NS, not significant (P>0.05). **(c)** T-bet green reporter (T-bet^{GFP}) KN2 mice in B6 and BALB/c background were intravenously injected 5μg of α-GalCer and analyzed 3 hours later for cytokine secretion. Representative data from three independent experiments are shown.



Supplementary Figure 3. *i*NKT cell subsets in T-bet deficient mice. **(a and b)** Representative flow cytometry profile of thymic *i*NKT cells from 12 week-old mice and statistical analysis for 10-14 week-old mice using B6 WT (n = 13) *Tbx21*$^{+/-}$ (n = 16) and *Tbx21*$^{-/-}$ (n = 5) mice are shown. Pooled data from three independent experiments. **(c)** Mixed bone marrow chimeras were generated with equal or unequal ratio of donor bone marrow cells using B6 WT and B6 *Tbx21*$^{-/-}$ mice. Six weeks later mice were sacrificed and analyzed *i*NKT phenotype. **(d)** Statistical analysis of WT or *Tbx21*$^{-/-}$ *i*NKT cells in 6 week-old BM chimeras (n = 4 ~ 5). Numbers indicate percentages of cells among total *i*NKT cells of WT or *Tbx21*$^{-/-}$ donor. Each Line indicates individual mice and paired two tailed *t*-tests were used. **(e)** Frequency of Eomes^{+} or CD44^{hi}CXCR3^{+} cells among CD8SP thymocytes are shown for 6 week-old B6 WT (n = 15) *Tbx21*$^{+/-}$ (n = 15), and *Tbx21*$^{-/-}$ (n = 4) mice. Pooled data from five independent experiments. **(f)** Frequencies of Eomes^{+} or CD44^{hi}CXCR3^{+} cells among CD8SP thymocytes from WT (n = 4) *Tbx21*$^{+/-}$ (n = 5), and *Tbx21*$^{+/-}$ *Cd1d*$^{-/-}$ (n = 3) CB6 mice are shown. Pooled data from two independent experiments. **(g)** Representative flow cytometry profile of CD8SP thymocytes from CB6 mice of indicated genotypes are shown. $^{***}P$<0.001, $^{**}P$<0.01, $^{*}P$<0.05; NS, not significant (P>0.05). Each symbol represents an individual mouse and one-way ANOVA was used for analysis (a, b, e and f).

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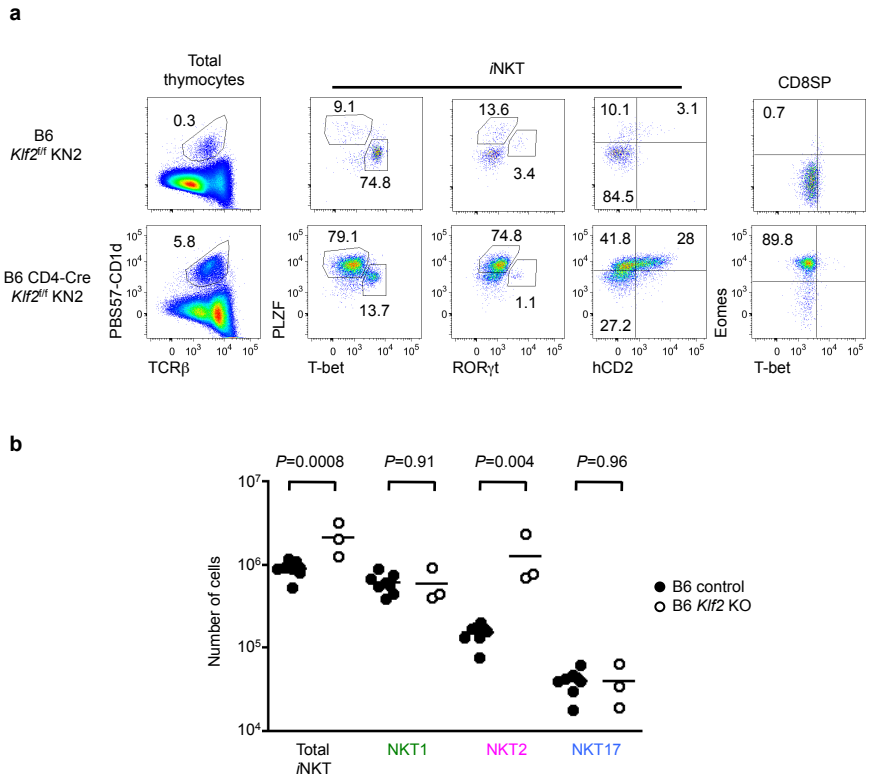

Supplementary Figure 4. KLF2 deficiency facilitates the development of NKT2 cells. **(a)** Thymic *i*NKT cells from 6 week-old KLF2 deficient and littermate control mice were analyzed for PLZF, T-bet and RORγt expression. **(b)** Numerical analysis of *i*NKT subsets of WT (n = 8) and *Klf2*$^{-/-}$ (n = 3) B6 mice is shown. Unpaired two tailed t-tests were used to compare each subset. Pooled result of three independent experiments.

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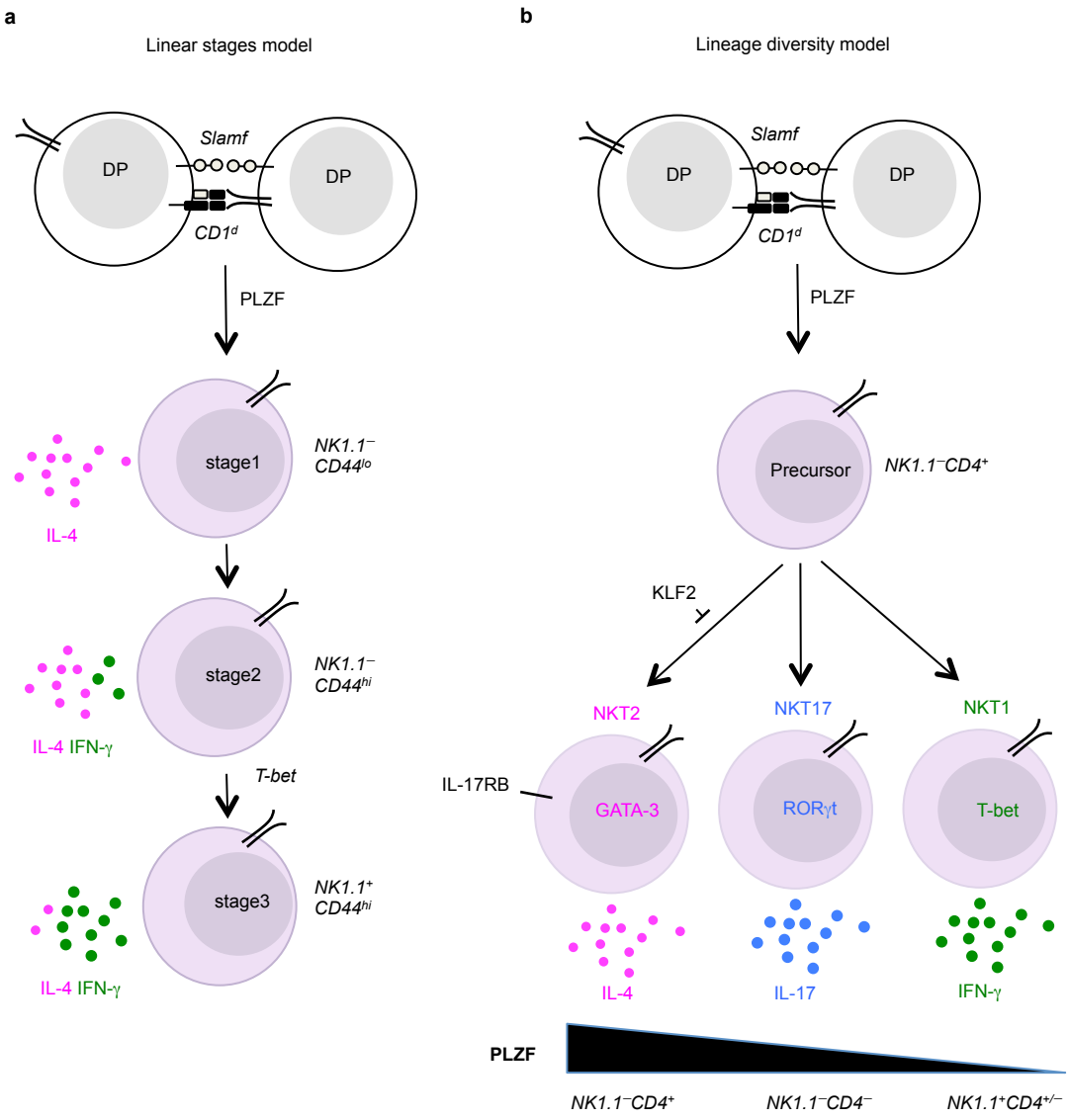
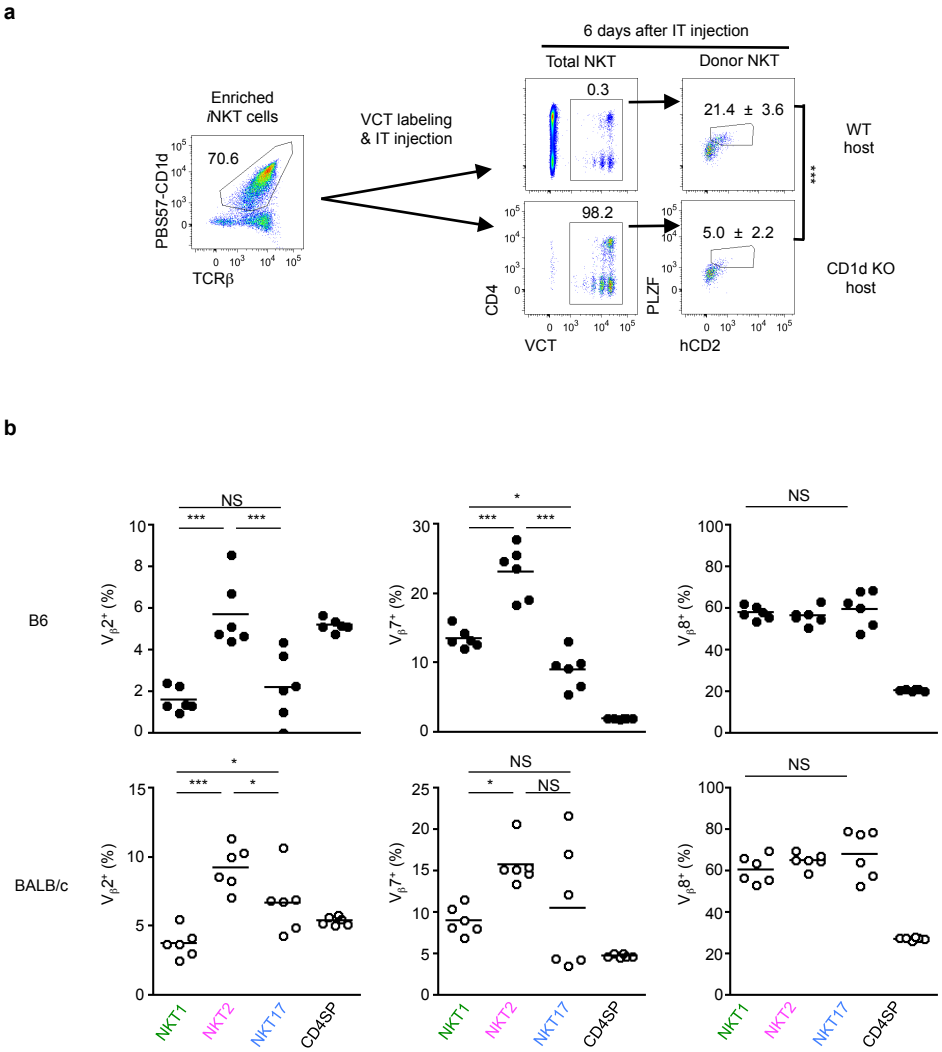


Supplementary Figure 5. Revised model for *i*NKT cell differentiation. **(a)** The currently held "linear stages" model supports a linear differentiation of *i*NKT cells from immature stage 1 cells to mature stage 3 cells. **(b)** Our data support a new transcription factor based model of *i*NKT subsets, where terminally differentiated cells producing distinct cytokines derive from a common precursor.



Supplementary Figure 6. IL-4 producing *i*NKT cells are stimulated by self ligands. **(a)** Thymic *i*NKT cells from BALB/c KN2 mice were MACS enriched by depleting CD8 and CD24 positive cells, labeled with violet cell tracer (VCT) and intrathymically (IT) injected into WT or CD1d KO BALB/c hosts. Six days later, VCT positive donor *i*NKT cells were analyzed after enrichment of CD1d tetramer positive cells. Gates in right panel show percentage of PLZF^{hi} NKT2 cells expressing hCD2. Unpaired two tailed *t*-tests were used to compare frequency of NKT2 cells in WT (n = 5) or *CD1d*$^{-/-}$ (n = 5) hosts. **(b)** Six to seven weeks old B6 (n = 6) and BALB/c (n = 6) mice were analyzed for TCR V_β 2, 7 and 8 repertoire by flow cytometry. One-way ANOVA was used to compare the frequency of NKT1, NKT2 and NKT17 cells. Pooled results from two independent experiments. *** *P<0.001*, ***P<0.01*, * *P<0.05*. NS, not significant (*P*>0.05).

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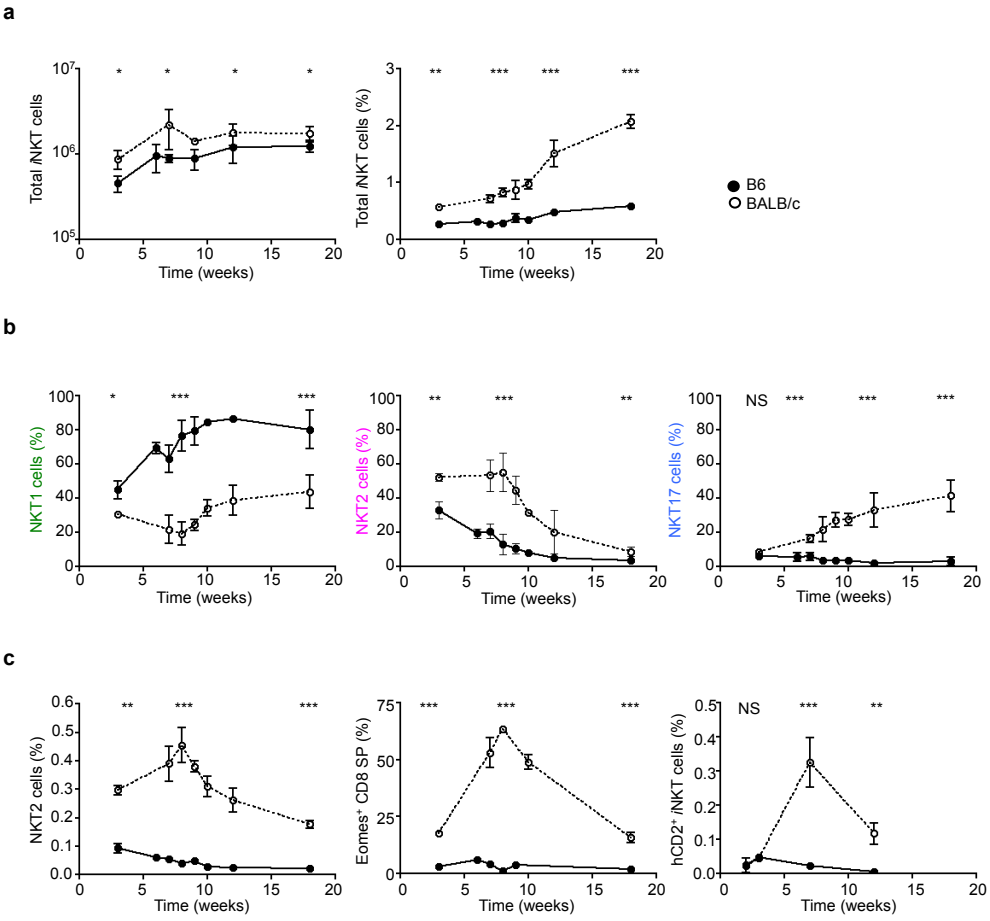

Supplementary Figure 7. Age-dependent kinetics of NKT1, NKT2 and NKT17 lineages in B6 and BALB/c thymi. **(a)** Number (left panel) and frequency among total thymocytes (right panel) of *i*NKT cells are shown. **(b)** Percentages of each *i*NKT subset among total *i*NKT cells are shown. **(c)** Frequency of NKT2 cells among total thymocytes (left), Eomes$^+$ cells among CD8SP thymocytes (middle) and hCD2$^+$ *i*NKT cells among total thymocytes (right) are shown. Data are represented as mean +/− SD at each time point. Pooled data of 12 independent experiments with 3~9 mice in each time point and a total of 38 B6 and 35 BALB/c mice. Unpaired two tailed *t*-tests were used to compare indicated time points. ***P<0.001, **P<0.01, * P<0.05; NS, not significant (P>0.05).